Original Research Report

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Effect of Desiccating Stress on Mouse Meibomian Gland Function

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ABSTRACT **Purpose: Mice exposed to standardized desiccating environmental stress to induce dry eye-like symptoms have been used as a model to study the underlying mechanisms of evaporative dry eye. While studies have shown marked inflammatory and immune changes, the effect of such stress on meibomian gland function remains largely unknown. We sought to evaluate the effects of desiccating stress on meibocyte proliferation and meibum quality. Methods: Ten mice were treated with scopolamine and subjected to a drafty low humidity environment (30-35%). Five and ten days after treatment, eyelids were harvested and cryosections stained with Ki67 antibody to identify cycling cells. Sections were also imaged using stimulated Raman scattering (SRS) microscopy to characterize the gland compositional changes by detecting the vibrational signatures of methylene (lipid) and amide-I (protein). Results: Desiccating stress caused a 3-fold increase in basal acinar cell proliferation from 18.3 ± 11.1% in untreated mice to 64.4 ± 19.9% and 66.6 ± 13.4% after 5 and 10 days exposure, respectively ($P < .001$). In addition, SRS analysis showed a wider variation in the protein-to-lipid ratio throughout the gland, suggesting alterations in meibocyte differentiation and lipid synthesis. Conclusions: These data are consistent with a model that a desiccating environment may have a direct effect on meibomian gland function, leading to a significant increase in basal acinar cell proliferation, abnormal meibocyte differentiation, and altered lipid production.**

KEY WORDS evaporative dry eye, meibomian gland, nonlinear optical microscopy, stimulated Raman scattering

I. INTRODUCTION

Meibomian gland dysfunction (**MGD**) is the leading cause of dry eye disease,[1] which affects an estimated 21 million people in the United States alone.[2] Chronic dry eye, when left untreated, can lead to ophthalmic complications such as impaired vision and increased vulnerability to eye infections.[3] Consequently, a better understanding of the progression of MGD may facilitate the development of effective therapeutic strategies against dry eye disease. In particular, comparative analysis of structural and biochemical features in normal and dysfunctional glands may reveal important insights into the pathophysiology of MGD.

Based on studies of dry eye patients and animal models, it has been noted that dry eye symptoms are accompanied by changes in meibomian gland structure, as well as in quality and quantity of glandular lipid secretion.[4,5] In particular, alteration in meibum quality is likely to be a significant marker for MGD progression.[6] In this regard, while structural abnormality such as terminal gland obstruction can be detected from excised eyelid by using H&E staining and standard optical microscopy,[4,7,8] analyses of meibum have been primarily conducted using samples that are secreted or extracted from the gland.[9-11] Without information pertaining to the gland structure, the mechanism that underpins meibum modification within dysfunctional glands cannot be directly observed. For example, it is unclear whether changes in meibum quality and quantity are a consequence of defective meibocytes, plugging of the duct, or other unknown phenomena.[4,12] It has been suggested that meibum

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OUTLINE

viscosity in MGD may increase due to the accumulation of protein,[13] such as from cellular materials being sloughed off from the thickened epithelium.[4] To further our understanding of MGD and dry eye disease, analysis of meibum content in the context of the glandular structure is essential.

Although characterizing meibum within the gland is important, it remains a challenging task. Common staining protocols such as H&E are generally unsuitable to study lipid-rich meibum, which readily dissolves in alcohol-based solvent.[4,14] To our knowledge, there is no standardized protocol to assess the chemical makeup of meibum at different functional parts of the gland. A tool that is able to visualize and quantify lipid as well as protein-rich cellular materials in meibum within intact gland can potentially provide important clues to the mechanism that renders meibomian glands dysfunctional. Ideally, such a tool should also permit the application of other analytical methods such as immunohistochemistry and commonly used optical microscopy.

In recent years, stimulated Raman scattering (**SRS**) microscopy has been gaining popularity for label-free imaging of biological systems.[15-17] Similar to second-harmonic generation (**SHG**) microscopy, SRS is a nonlinear optical technique in which the signal is not dependent on exogenous labels and does not require destructive sample preparation. SRS signals are derived from the molecular vibrations in the focal spot. By tuning the frequency of the excitation beams, different vibrational modes can be probed, enabling selective visualization of tissue components of interest. For example, the utility of SRS to detect carbon-hydrogen vibrations of lipid and cholesterol has been established for studies of fixed specimen, as well as live animals.[18-20] Protein-rich materials can also be visualized by vibrationally tuning into protein-specific chemical groups such as the amide moiety.

In MGD, several studies have identified changes in relative quantities of protein-to-lipid (**P/L**) within expressed meibum and hypothesized the importance of having a specific P/L ratio.[4,21-23] In the current study, we used the chemical group selectivity of SRS microscopy to quantify P/L ratios within the meibomian gland. Specifically, we combined immunofluorescence and label-free SRS microscopy to identify changes in meibomian glands of a dry eye mouse model. The dry eye mouse model has been recently developed using desiccating stress consisting of low humidity (30-35%), continuous airflow, and inhibition of tear secretion by systemic scopolamine administration.[24,25] Affected mice show ocular surface features that mimic evaporative dry eye in human; however, the response of the meibomian gland to such environmental stress has not been previously evaluated.

In this study, we monitored Ki-67 antigen staining to assess meibocyte proliferation and characterized meibum composition in different parts of the gland using SRS. We report for the first time that desiccating stress induced meibocyte cell cycle entry, suggesting abnormally high meibocyte proliferation and downstream meibum production in response to ocular surface desiccation. Furthermore, we show for the first time that while the protein content of normal meibum progressively decreases from the acini to orifice, suggesting meibum maturation, desiccating stress blocks maturation and leads to increased protein content within the meibum lipid. Based on these findings we propose that desiccating stress may induce MGD through altered meibocyte differentiation leading to changes in meibum quality.

II. MATERIALS AND METHODS

A. Mouse Model and Tissue Preparation

A total of ten adult female mice, 8 weeks old, (C57BL/6) were subjected to standardized desiccating stress conditions at the Ocular Surface Center, Baylor College of Medicine, as previously described.[26,27] Mice were sacrificed after 5 and 10 days (n=5 and n=5, respectively). Untreated adult mice (n=5) were utilized for the control group. Exenterated eyes with intact eyelids were collected, embedded in OCT, cryosectioned (10 μm thick sections) and placed on standard microscope slides. Slides were then shipped on dry ice to the Gavin Herbert Eye Institute for immunofluorescence and SRS microscopy. Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee protocol at Baylor College of Medicine and adhered to the ARVO Resolution on the Use of Animals in Vision Research.

B. Immunofluorescence Microscopy

Sections were air dried for 20 min, rehydrated in PBS (3 × 5 min) and blocked with 10% goat serum at 37°C for 30 min. Anti-Ki67 (Abcam; 1mg/mL, 1/500 dilution) was applied to the tissue sections for overnight at 4°C. Sections were then washed in PBS (3 × 5 min) and secondary antibody (Goat Anti-Rabbit AlexaFluor546; 1/1000 dilution) was applied to the sections for 1 hour at 37°C. Subsequently, sections were rinsed with PBS (3 × 5 min), counterstained with DAPI (1:5000) and then mounted with a coverslip. Fluorescent imaging was performed on a Leica DMI6000B using a 20×/0.75NA objective. Digital images from both the upper and lower lid were taken from all 15 mouse